Chapter 3

Effect of Preheat Temperature on the Hydrophobic Properties of Milk Proteins

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Pre-heat treatment of skim milk for the preparation of nonfat dry milk (NDM) powders results in physicochemical changes that effect the functional behavior of the powder. The functional properties of NDM powders heated to 63°C (I), 74°C (II), and 85°C (III) for 30 min before spray drying were different. Comparison of reversed phase chromatographic profiles indicated that milk proteins were altered and a whey-casein complex was formed in II and III. Hydrophobic interaction chromatography (HIC) indicated that the complex formed eluted before ß-casein and hence was more hydrophilic than was indicated under reversed phase chromatography conditions. Alkane binding to the milk proteins in the rehydrated powders also indicated that II and III were more hydrophilic than I. Electron micrographs of immuno-gold labeled whey proteins in the rehydrated powders indicated that less than ten percent of the gold particles were associated with the casein micelles for I but greater than fifty percent were associated with the micelles for II and III. The appearance of the complex formed in II and III on the surface of casein micelles suggests it may contribute to the increased hydrophilic character of these powders. Examination of their functional properties indicated that III had the lowest solubility and the greatest percent overrun in a foaming test. The emulsifying activity index (EAI) was greatest for II and no significant differences were observed for foam and emulsion stability between the three powders.

Nonfat dry milk (NDM) is frequently used as a functional ingredient for dairy, bakery, confectionery, and other food applications. Heat treatment of skim milk before spray drying is used widely as a means of manipulating the functional properties of NDM powders in milk products. As a result, the end-use of such products is very dependent on the heat treatment the powders receive (1). Heat treatments can denature whey proteins resulting in aggregation which alter the ability of the powders to rehydrate. Although difficult to distinguish, aggregation is a separate and usually irreversible process which follows denaturation of whey protein. Denaturation is normally reversible and can be stopped before aggregation begins (2). Limited whey protein denaturation can improve the emulsifying properties of whey protein in food systems (3). Caseins, which account for about 80% of the total milk proteins, exist in milk as soluble complexes or micelles. Caseins are amphiphilic in nature and are primarily responsible for the excellent surfactant properties or functionality of milk ingredients. Unlike whey proteins the caseinate system in milk is very stable to heat and tends to resist coagulation. Heat treatment, however, is known to increase the acidity of milks, primarily through the decomposition of lactose, and also to reduce both total soluble and ionic calcium resulting in flocculation of casein micelles and a corresponding loss of functional properties.

Preheat treatment of skim milk between 85°C and 100°C for 30 min in the preparation of NDM powders is commonly used in the baking industry to improve the extensibility and water absorption of dough (4). High-heat NDM powders (85°C) absorb more water than lower heated powders and this can improve its emulsion stability and gelation properties (5). Such functional properties are important in comminuted meat, confectionery, as well as in reconstituted concentrated sterile and baked products. Generally extensive denaturation is used to prevent undesired side reactions such as loaf depression or coagulation. Because some water absorption is necessary, ice cream and dairy beverages are frequently made with low- and medium-heat NDM (63°C, 74°C). Yogurt texture is greatly affected by the degree of whey protein denaturation (6). Batch-type heating of skim milk between 85-95°C for 5 to 10 min before inoculation is an important processing variable in the manufacture of yogurt.

The purpose of this study was to assess the heat induced physicochemical changes that occur during manufacturing of NDM powders. Changes in milk protein profiles were identified using chromatographic methods

and micellar associations of whey proteins or complexes were examined by immuno-labeled electron microscopic techniques.

Material and Methods

Nonfat Dry Milk. Raw pooled herd milk from Holstein, Ayrshire, and Brown Swiss cows was skimmed at 39°C and preheated at 63°C, 74°C, and 85°C for 30 min; concentrated; then spray dried to yield NDM powders I, II, and III according to a previously published procedure (7).

Hydrophobic Interaction Chromatography (HIC). Chromatographic separations of milk proteins were carried out on a Spectra Physics (San Jose, CA) SP-8800 HPLC system, equipped with a LC-HINT column, 0.46 x 10 cm, (Supelco, Inc., Bellefonte, PA); mobile phase, solvent B: 0.05M sodium phosphate, pH 6.0, in 3.75M urea, solvent A: 2.0M ammonium sulfate in solvent B; gradient, 0%-100%B, 30 min; flow rate, 0.8 mL/min; detector gain 0.1 aufs. Sample, 1.0 g NDM was dissolved in 10 mL of water, and centrifuged at 100,000 x g, 4°C, 40 min. The supernatant was filtered (0.45 μM pore size) and 200 μL injected onto the column. The pellet, 3 mg, was dissolved in 1 ml of solvent B, H_2O, solvent A, separately, and 100 μL injected onto the column.

Gel Electrophoresis. SDS- and urea-PAGE of skim milk proteins was carried out on a PhastSystem (Pharmacia, Piscataway, NJ) as previously described (8 and Van Hekken, D. et al. J. Dairy Sci. in press). The molecular weight standards from Bio-Rad, (Richmond, CA) and their corresponding molecular weights for SDS-PAGE were: phosphorylase b, 97,400; bovine serum albumin (BSA), 66,200; ovalbumin, 42,699; carbonic anhydrase, 31,000; soybean trypsin inhibitor, 21,500; lysozyme, 14,000.

Electrophoretic Blotting. A modification of the method of Towbin et al. (9) was used for the electrophoretic transfer of milk proteins from the polyacrylamide gel to nitrocellulose (NC) membrane. Half of the gel containing the skim milk proteins (39°C) was stained with Coomassie R350 dye and the other half was removed from the cellophane backing for electroblotting. The gel isolated for immunoblotting was allowed to equilibrate in transfer buffer; 25mM Tris, 192mM glycine, and 20% methanol, pH 8.3, for 5 min. The proteins were transferred to a NC membrane using a Mini Trans-Blot cell (Bio-Rad, Richmond, CA) at 60 V, 0.15 A, for 3 h at room temperature with electrodes 8 cm apart.

Immunological Detection. To avoid nonspecific binding, the NC membrane was incubated in blocking buffer; 50mM Tris, 150mM NaCl, pH 7.4, containing 2.5 g gelatin and

2.5 mL 10% NP-40. The membrane was incubated for 1 h with primary immune antiserum to cow whey protein Cat. #AXL-306 (Accurate Chemical and Scientific Corp., Westbury, NY) and secondary antibody, goat antirabbit IgG conjugated to horse radish peroxidase (Behring Diagnostic, La Jolla, CA) for 1 h. Both antisera were diluted 1:1000 in blocking buffer. The peroxidase substrate was developed in 4-chloro-1-naphthol, 10 mg in 3.3 ml methanol 16.7 ml blocking buffer and 10 μL of 30% H_2O_2.

Electron Microscopy. Samples of heated skim milk and rehydrated NDM powders were prepared separately for immunolabeling as whole mounts by negative staining and as thin sections, embedded in plastic. For labeling with negative staining, aliquots of milk containing 0.1% gelatin were incubated for 1 h at room temperature with rabbit antiserum to cow whey protein in phosphate-buffered saline (PBS). The mixture was absorbed to Formvar-carbon coated Ni Specimen grids for 10 min, washed with 5-10 drops of 1% gelatin in PBS, and incubated for 1 h on a 100 μL drop of goat antirabbit IgG, conjugated to 10 nm colloidal gold particles GAR 10, (Jannsen Life Sciences Products, Beerse, Belgium). The samples were washed sequentially with: 1% gelatin in PBS, PBS, water, and finally stained with 2% uranyl acetate solution.

For immunolabeling of thin sections, fresh samples of milk were embedded in the low temperature embedment, Lowicryl K4M, according to the procedure of Altmann et al. (10). Thin sections on Formvar-carbon coated Ni specimen grids were incubated sequentially with: 1% gelatin in PBS, rabbit antiserum to cow whey protein, gelatin/PBS, and goat antirabbit IgG, conjugated to colloidal gold particles. Sections were then washed with gelatin/PBS, PBS, and water before post staining the sections with 2% uranyl acetate.

Hydrophobicity. Protein hydrophobicity was determined by measuring the extent of alkane binding to NDM protein by a modification of the procedure of Mangino et al. (11). Two mL of aqueous NDM solution (10%) and heptane (1.5 mL) were added to 5 mL vials, sealed with parafilm, and mixed sideways at 3 rev/min at 25.0 ± 0.2°C for 18 h with a model R-7636-00 Roto-torque rotator, (Cole-Palmer, International Chicago, IL, USA). The alkane layer was discarded and the aqueous phase (1.0 ml) was extracted with undecane (0.6 mL) containing an internal standard (octane) for 2 h at 3 rev/min. Samples (0.5 μL) of the undecane phase were injected into a model 5710A gas chromatograph (Hewlett-Packard Co., Palo Alto, CA) equipped with a flame-ionization detector. Separations were carried out on a column of Chromosorb W coated with 10% (w/w) OV-101.

Functional Properties. Solubility measurements were performed as previously described (7). Emulsions were prepared by homogenizing 3 mL of protein solution (10%) and 1 mL corn oil for 30 sec using a Polytron homogenizer at setting #5 and determining the emulsion activity index (EAI) by turbidity measurements at protein concentrations of 0.1, 0.5 and 1.0% and pH values of 6, 7, 8, and 9 according to the procedure of Pearce and Kinsella (12). Foam formation (% overrun) and foam stability were determined according to the procedure of Phillips et al. (13) with the modification that foam stability was determined by measuring the time required for drainage equal to 50% of the weight of foam (approximately 100 mL).

Results and Discussion

HPLC Separations. Earlier results have shown that elution profiles for soluble dialyzed material from rehydrated medium- and high-heat NDM powders (II and III, respectively) were significantly different from the low-heat powder (I) profiles (8). The profiles for I and III are depicted together with the SDS-PAGE patterns of the proteins present in corresponding peaks from the analytical column (inset) in Figure 1. The whey-casein complex formed in III was comprised of many proteins including BSA, κ-, and α_{s2}-caseins, ß-lactoglobulin (ß-LG), and α-lactalbumin (α-LA) (Figure 1b, lane 6). Although RP-HPLC profiles and SDS-PAGE gels of reduced proteins indicate that the complex is stabilized through disulfide linkage, some of the native whey proteins reformed during storage suggesting that whey complexes are also present which are stabilized through less specific interactions (hydrophobic or calcium-dependent linkages) with the casein micelles. Comparison of retention times for RP-HPLC separation of milk proteins from III indicated that the whey-casein complex is more hydrophobic under the conditions of chromatography than the other milk proteins including ß-casein (Figure 1b).

Elution of milk proteins from the same rehydrated powders using hydrophobic interaction chromatography (HIC) indicated that the whey-casein complex was less hydrophobic than ß-casein. The whey-casein complex was not present in the supernatant from I but was present in the elution profile of the supernatant from III and eluted before ß-casein (Figure 2). The complex was also found in the pellet from III and eluted before the caseins (Figure 3b). SDS-PAGE patterns of the whey-casein complex (left inset) Figure 3b, lane 1, indicated that the sample contained aggregates that did not enter the running gel. Casein micelles apparently were not completely dissociated in the HIC buffer since ß- and α_{s1}-caseins were present in the complex fraction along with κ-casein and the whey proteins (see identification of milk proteins Figure 1a). The second and third peaks

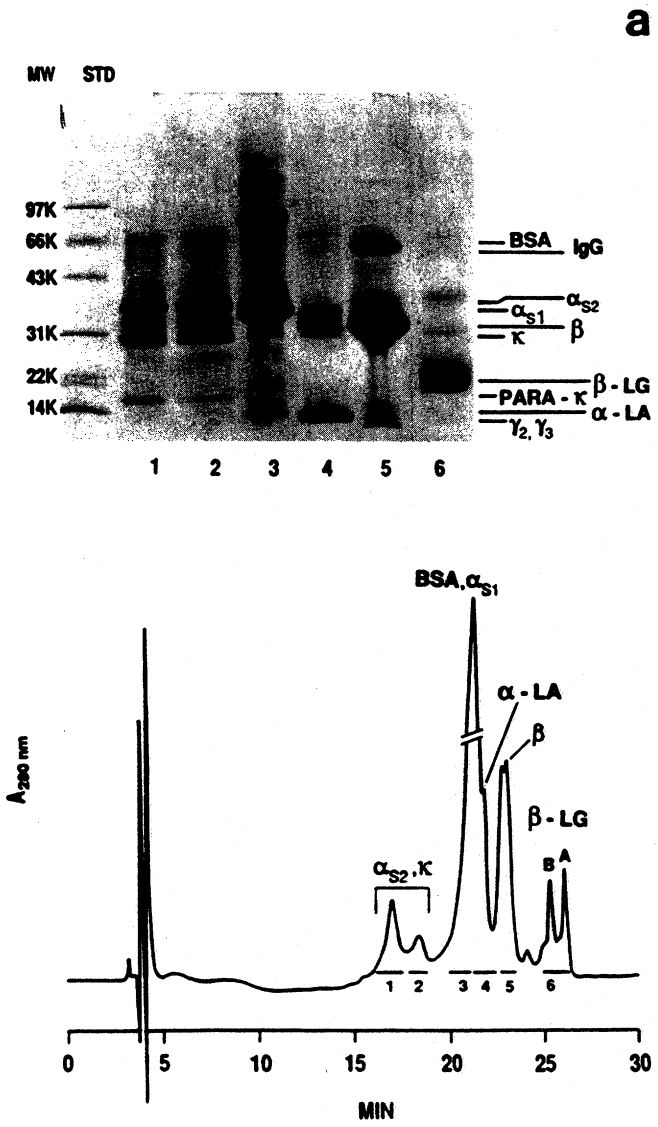

Figure 1. RP-HPLC elution profile and electrophoretic (SDS-PAGE) patterns of dialyzed soluble proteins from rehydrated NDM. a. I, b. III. (Reprinted from ref. 8. Copyright 1990 American Chemical Society.)

b

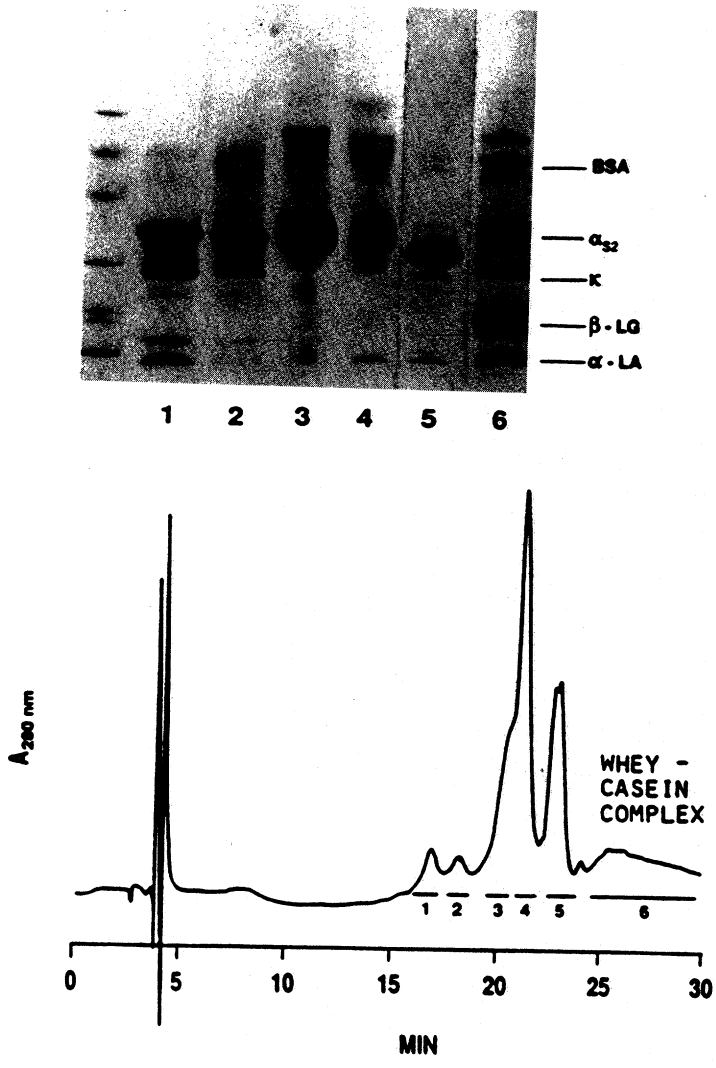

Figure 1. Continued.

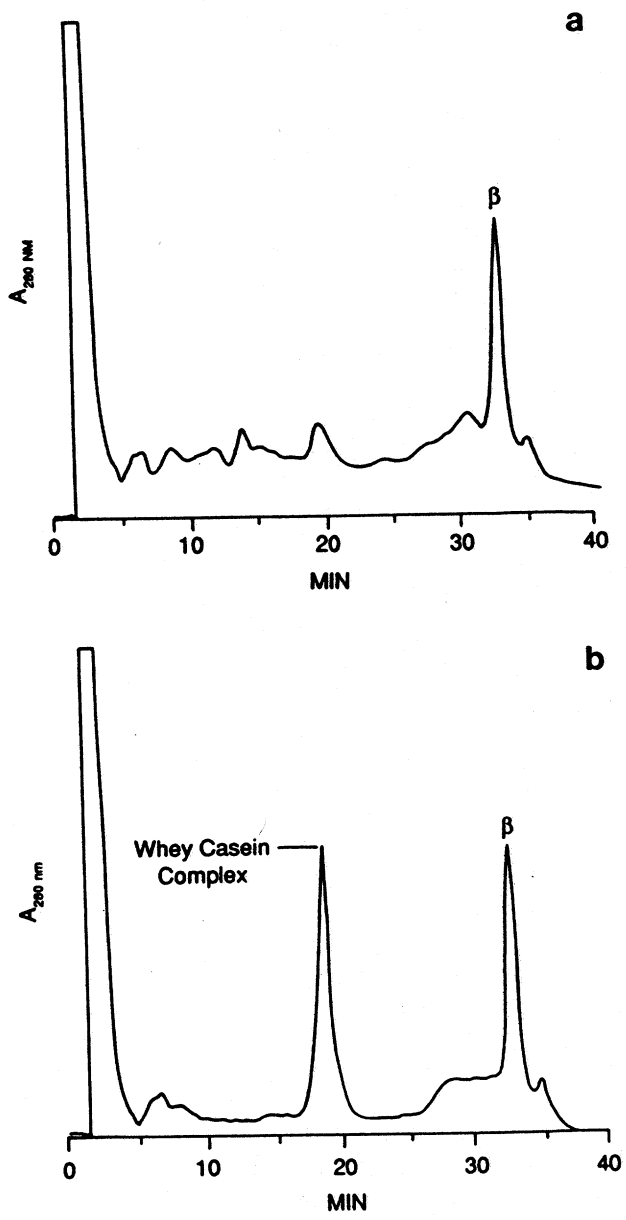

Figure 2. HIC elution profile of supernatant from rehydrated NDM powders. a. I, b. III.

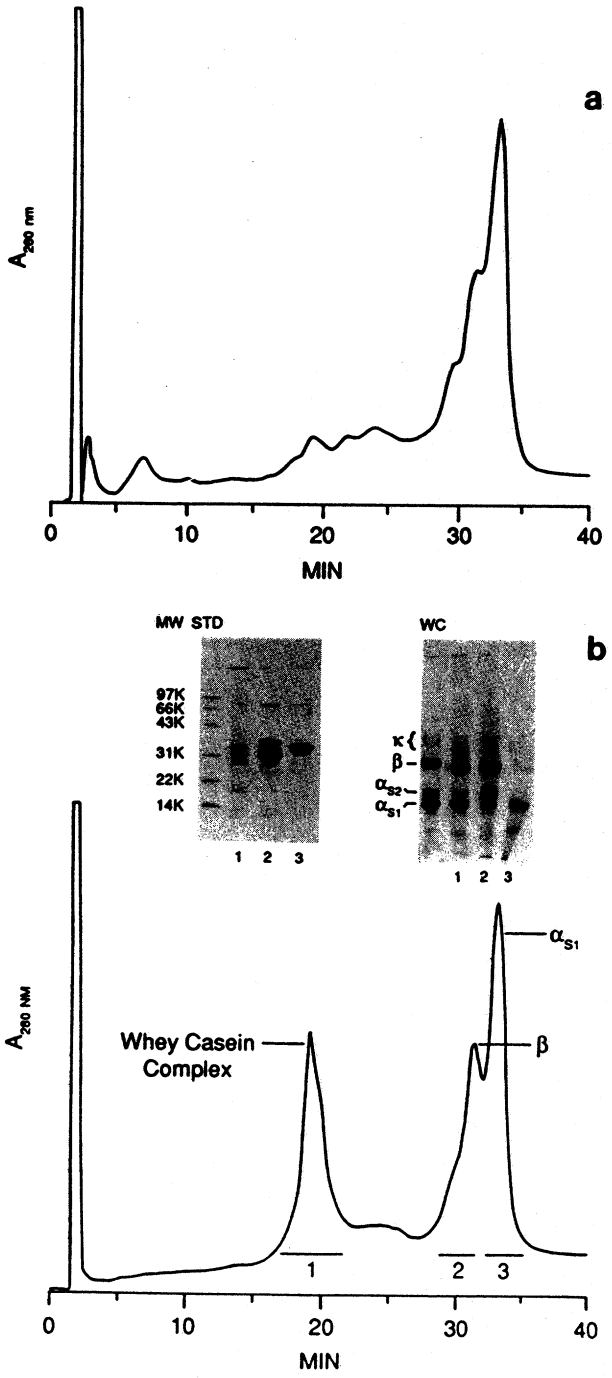

Figure 3. HIC elution profile and electrophoretic patterns of pellet from rehydrated NDM powders. a. I; b. III (inset, left = SDS-PAGE, right = urea-PAGE).

in the same figure were composed primarily of ß- and α_{s1}-caseins respectively (left) inset lanes 2 and 3). Caseins in whole casein (WC) can be identified more easily with urea-PAGE (Figure 3, right inset). Urea-PAGE of the whey-casein complex (right inset, lane 1) clearly shows the presence of α_{s2}-casein, which was not detected by SDS-PAGE, probably because of its poorer solubility in that chromatographic system. Chromatographic conditions used for HIC of the whey-casein complex formed on heating skim milk before spray drying, therefore, indicate that the complex is less hydrophobic than previously indicated by RP-HPLC.

Heptane Binding. The amount of heptane bound to the proteins for the three powders decreased with increasing preheat temperature (Table I). Although the difference between I and II was not statistically significant there was a significant difference in alkane binding between I and III. This could be attributed to the loss of ß-lactoglobulin's alkane binding site to denaturation however charged residues for most proteins are preferentially located at the protein surface, hydrophobic groups buried within the protein away from its surface. Heat denaturation should therefore expose more hydrophobic residues; bind more alkane and improve functional behavior.

Table I. Alkane binding to rehydrated NDM powders

NDM	Heptane bound (mg/g protein)
LOW (I)	162 ± 9
MEDIUM (II)	144 ± 10
HIGH (III)	139 ± 4

n = 8

Location of Whey Protein Complexes. In order to better understand the spacial relationship between the whey-casein complex formed on heating milk and the casein micelle, whey proteins in samples were immunolabeled with colloidal gold and examined by electron microscopy. A western blot of immune antiserum to cow whey proteins was run against skim milk (39°C) to determine antibody specificity. SDS-PAGE separation of milk proteins is shown in Figure 4 (left half) along with an electroblot of the same proteins onto the NC membrane (right half). Results indicated that immune antiserum bound to BSA and α-LA; but binding to IgG, ß-LG and the caseins was weaker. Electron micrographs of immuno-gold labeled, negative

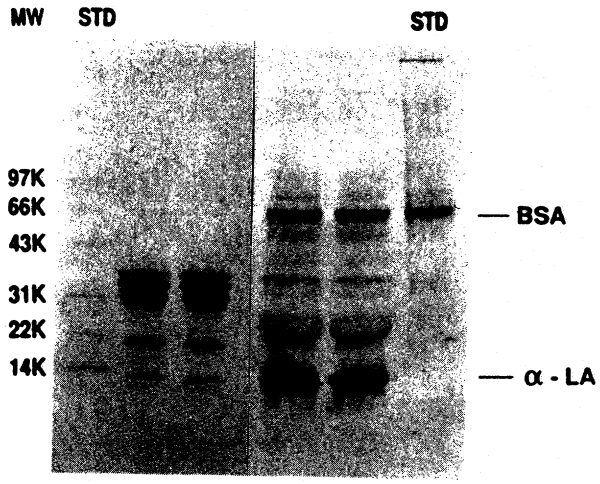

Figure 4. Western blot of antiserum to cow whey protein against milk (39°C). Left half, SDS-PAGE Gel; Right half, NC membrane

stained, heated skim milk samples indicated little or no label associated directly with the surfaces of micelles at 39°C, instead a few isolated gold particles were located in spaces between micelles (Figure 5a). However, (Figure 5b) the surfaces of many micelles from milks heated at 85°C were labeled with several colloidal gold particles. Label was primarily associated with material on or near the surfaces of micelles (arrows), although some isolated particles and small groups of gold label were located in areas between micelles. For post-embedment immuno-labeling of thin sections at 39°C, (Figure 5c) some isolated particles of colloidal gold label were located on or near the surfaces of micelles. At 85°C, (Figure 5d) numerous particles of gold label were found either singly or, more often, in clusters, mostly in areas related to the (cut) surface of micelles (arrows). Electron micrographs of immuno-gold labeled negative stained rehydrated NDM powders, (Figure 6) indicated that most of the labeled particles were found in the areas between micelles for I, whereas most of the particles were associated with the irregular surface of the micelles for II and III (arrows). To determine quantitatively the location of the gold particles in the rehydrated milk samples, 100 particles were counted in each of the negative stained preparations. Greater than 50% of the particles were associated with micelles for II and III and less than 10% for I (see bar graph, Figure 6). Although several conflicting models have been proposed describing the structure of the casein micelle, most current models put κ-casein on the casein micelle surface. This model allows denatured serum proteins to interact with κ-casein on the micelle surface with possible interconnection of the micelles. Our results indicate that neither the heated skim milk (Figure 5d) nor III (Figure 6c) show significant linking of micelles through labeled whey proteins.

Functional Properties. The functional properties of the rehydrated NDM powders that were examined were solubility, emulsifying activity index (EAI), overrun, foam and emulsion stability. Isolated insoluble protein from rehydrated NDM subjected to 3 different processing temperatures was relatively small, (between 2-6% of the total protein in the powders) and was greatest for III. Insolubles in III also contained more lactose which has been suggested to interact with protein to form larger aggregates that coagulate (3). The EAI for the three powders, measured at protein concentrations of 0.1, 0.5, and 1.0% and at pH values of 6, 7, 8, 9, was greatest at 0.1% and pH 9 (Figure 7). Regardless of concentration and pH there was a consistent increase in emulsifying activity for II followed by a significant decrease for III. Since it was determined that approximately 50 and 95% of the whey protein in II and III respectively was denatured,

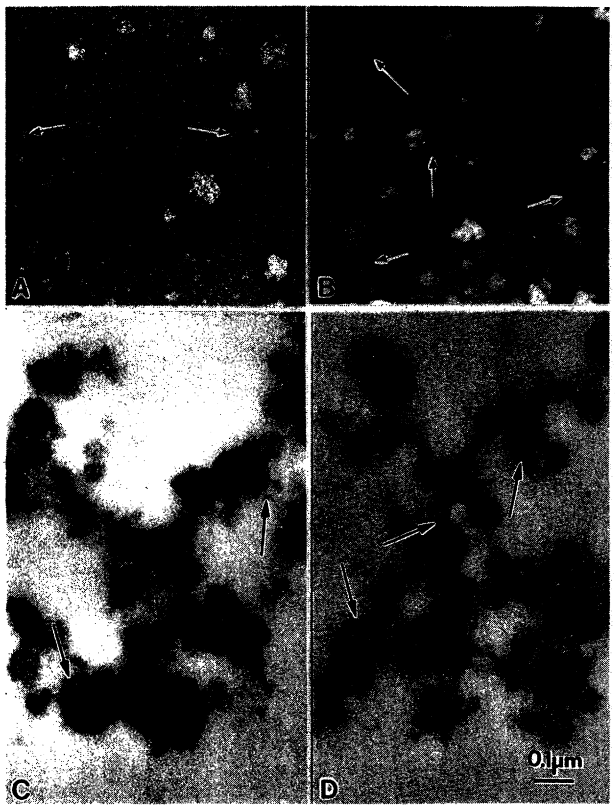

Figure 5. Electron micrographs of immuno-gold labeled skim milk. Heat treatment and preparation: A. 39 °C, negative stained; B. 85 °C, negative stained; C. 39 °C, thin sectioned; D. 85 °C, thin sectioned. Labeled whey protein (arrows).

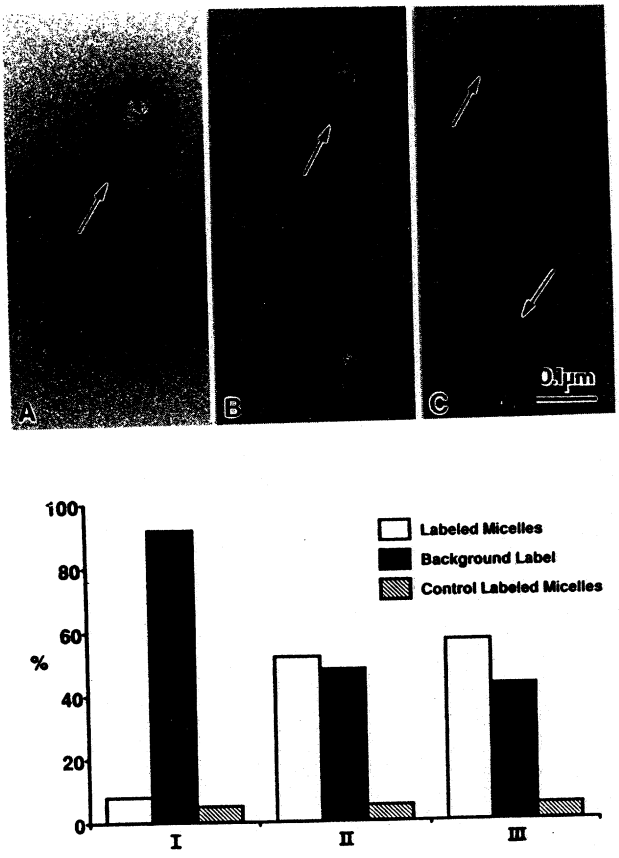

Figure 6. Electron micrographs of immuno-gold labeled rehydrated NDM powders. A. I, B. II, C. III; Negative stained. Labeled whey protein (arrows). Bar graph represents micellar association with particles (control contains no primary antibody).

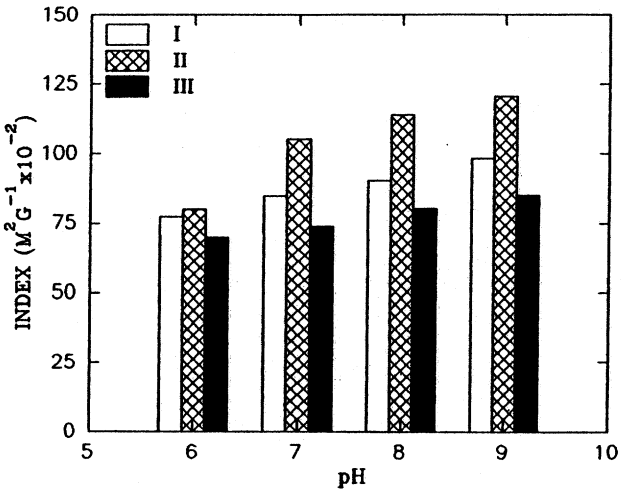

Figure 7. Emulsifying activity index for NDM powders 0.1% (w/v).

the greater EAI for II could be interpreted as the results of limited whey protein denaturation where unfolding of protein molecules exposes hydrophobic groups that can more easily orient at the oil-water interface and improve emulsifying properties. Mottar et al. (6) have suggested that further heating results in a decrease in the ratio of ß-LG: α-LA associated with the casein micelle; an increase in water holding capacity and lower surface hydrophobicity. In general, mild heat treatment reduces whipping times, increases overrun, and enhances foam stability. For the rehydrated powders the overrun formed on whipping was consistently greater for III (Figure 8) however, little difference in foam stability was observed between the three powders (Figure 9).

Conclusions. This study demonstrates that preheat treatment of skim milk alters the micelle surface and effects the hydrophobic properties of NDM powders. Using various chromatographic and electron microscopic techniques the formation, composition, and location of the whey-casein complex in heated skim milk was elucidated. Of the functional properties evaluated, the emulsifying activity was the most interesting. Regardless of protein concentration and pH, the EAI was consistently higher for II. Although limited denaturation of milk proteins has been suggested to explain the greater emulsifying ability of II compared to I or III, further research is required to demonstrate that the increase is a result of protein localization at the oil-water interface. In addition, the embedding procedure chosen for EM was selected for

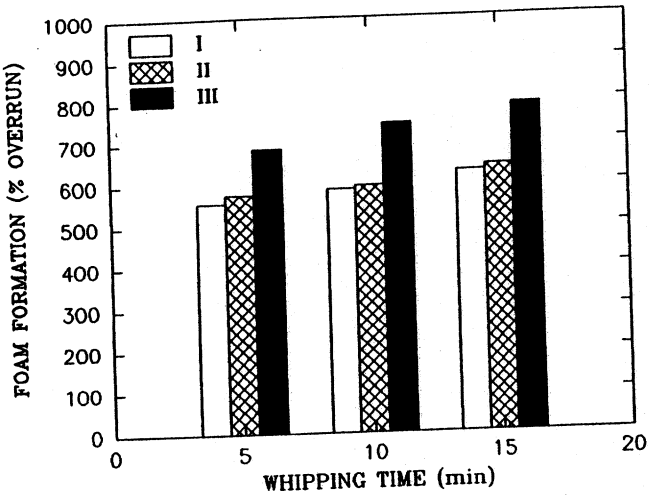

Figure 8. Foam formation (% overrun) for NDM powders.

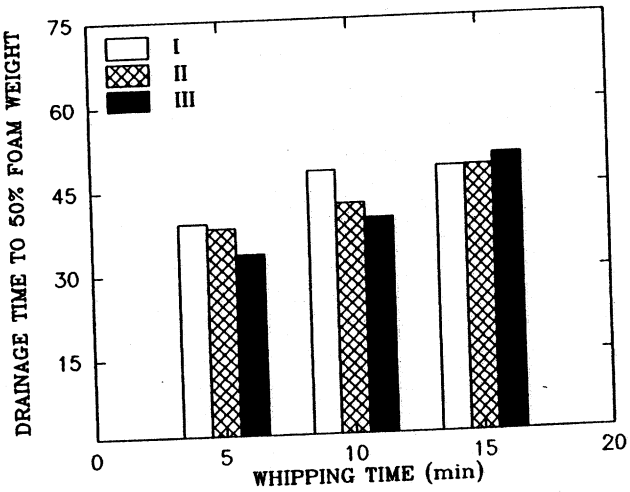

Figure 9. Foam Stability of NDM powders.

structural preservation and enhanced immunolabelling of the samples however the location of κ-, and α_{s2}-caseins in the micelle of heated and unheated milks was not determined. These two research areas warrant further investigation.

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Literature Cited

1. Pallansch, M. J. In By-Products from Milk; Webb, B. H., Whittier, E. O., Eds.; AVI Publishing Co.: Westport, CT, 1970; Chapter 5.
2. Brown, R. J. In Fundamentals of Dairy Chemistry; Wong, N. P., Ed.; Van Nostrand Reinhold Co: New York, 1988; Chapter 11.
3. Morr, C. V. In Functionality and Protein Structure; Pour-El, A., Ed.; ACS Symposium Series No. 92; American Chemical Society: Washington, DC, 1979; pp 65-79.
4. Guy, E. J. In By-Products from Milk; Webb, B. H., Whittier, E. O., Eds.; AVI Publishing Co.: Westport, CT, 1970; Chapter 7.
5. Kinsella, J. E. CRC Crit. Rev. Food Sci. Nutr. 1984, 21, 197-262.
6. Mottar, J.; Bassier, A.; Joniau, M.; Baert, J. J. Dairy Sci. 1989, 72, 2247-2256.
7. Parris, N.; Barford, R. A.; White, A. E.; Mozersky, S. M. J. Food Sci. 1989, 54, 1218-1221.
8. Parris, N.; White, A. E.; Farrell, H. M., Jr. J. Agric. Food Chem. 1990, 38, 824-829.
9. Towbin, H.; Staehelin, T; Gordon, J. Proc. Natl. Sci. USA 1979, 76, 4350-4354.
10. Altman, L. W.; Schneider, B. G.; Papermaster, D. S. J. Histochemistry and Cytochemistry 1984, 32, 1217-1223.
11. Mangino, M. E.; Fritsch, D.; Liao, S. Y.; Fayerman, A. M.; Harper, W. J. N. Z. J. Dairy Sci. Technol. 1985, 20, 103-107.
12. Pearce, K. N.; Kinsella, J. E. J. Agric. Food Chem. 1978, 26, 716-725.
13. Phillips, L.; Haque, Z.; Kinsella, J. E. J. Food Sci. 1987, 52, 1074-1079.